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Atomic force microscopy evidences of bacterial cell damage caused by propolis extracts on *E. coli* and *S. aureus*

Jessica Valéria de CAMPOS[1,2], Odílio Benedito Garrido ASSIS[2]*, Rubens BERNARDES-FILHO[2]

Abstract

The interaction of the aqueous extract of green propolis with the bacterial envelope was investigated using atomic force microscopy on *E. coli* and *S. aureus*, as Gram-negative and Gram-positive model microorganisms. The bacteria were incubated in the extracts with variable proportions of water/ethanol, i.e., in different concentrations of total phenolic and flavonoids, for 4 and 12 hours. All extracts exhibited antibacterial action with higher activity (lower MIC) for full ethanolic extract (100% alcohol) against both strains, what was attributed to a synergistic interaction of ethanol and phenolic compounds presented in greater concentration in this extract. According to AFM analysis, it was evidenced the propolis main mechanism of action as rupture and lysis of bacterial cells. Changes in bacterial morphology following treatment indicate membrane imbalances with the internal contents leaking out accompanied by cell swelling, due to possible water uptake through cell lysis. The intensity of such damage was found to be time and specie dependent.

Keywords: propolis; antimicrobial activity; bacterial wall integrity.

Practical Application: Clarification of the antimicrobial mechanism and the development of proper propolis extracts for uses as inhibitor agent of foodborne pathogens proliferation.

1 Introduction

Propolis is a well-known natural compound collected by bees from plant fluids and exudates and is used to protect and seal parts of the hive. Raw propolis consists essentially of resin (around 50% of de mass) and wax (30%), with fractions of essential oils (10%), pollen (5%) and several other organic-derived compounds and impurities (Pietta et al., 2002). The proportion of these compounds however, is strongly dependent on botanic source and geographic origin (Bankova et al., 2014).

Brazil is the third greatest producer of propolis in the world (after Russia and China) with a harvest estimated to be 100 ton/years (corresponding to 10-15% of worldwide production) (Machado et al., 2012), mostly of them (around 60%) destined to exportation (Inoue et al., 2007). Due to its large tropical expansion and wide variety of flora, Brazilian propolis is one of the most diversified and used in the world (Berretta et al., 2017).

Owing to its known health benefits, propolis has been used for centuries in popular medicine such as anti-inflammatory, antiviral, immunostimulant, anaesthetic and in the treatment of several other minor ailments (De Groot, 2013). The composition of propolis compounds however is quite complex and uncertain, with reports between 160 (Mirzoeva et al., 1997) to more than 200 chemical constituents (Kimoto et al., 1998). Amongst these, phenolic and flavonoid compounds are reputed to have the highest biological and pharmacological activities (Vennat et al., 1995; Marcucci et al., 2001).

These substances are soluble in an aqueous or alcoholic medium, thus liquid extracts are the most common form for human usage, either by oral administration or topic application. Within the literature there are several reviews describing the processes of propolis extract preparation, as well as application and characterization of their biological activities (Mirzoeva et al., 1997; Burdock, 1998; Bankova et al., 2014; Kubiliene et al., 2015).

Models have suggested that propolis antimicrobial activity results from the binding of some constituents to proteins cell walls, followed by adsorption or damage induced in the bacterial envelope (Burdock, 1998; Schnitzler et al., 2010).

Therefore, the aims of this study are: i) prepare aqueous and ethanolic extract from green Brazilian propolis; ii) to identify the main phenolic constituents; iii) to determine their minimum bacterial inhibitory concentration; and iv) evaluate the effects of extracts exposing extracts to the bacteria cell walls using atomic force microscopy in an effort to help clarify the nature of the molecular interactions involved in propolis antibacterial activity.

2 Materials and methods

2.1 Propolis samples and extracts preparation

Crude samples of bee (*Apis mellifera*) propolis, popularly known as “green propolis” due to its predominant color, were kindly provided by Wenzel Indústria e Comércio de Produtos Apícolas Ltda (São Carlos, SP, Brazil). The raw propolis was collected from the apiary located in the city of Barbacena, Minas Gerais State,

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[1] *Programa de Pós-graduação em Biotecnologia, Universidade Federal de São Carlos – UFSCar, São Carlos, SP, Brasil*
[2] *Laboratório Nacional de Nanotecnologia para o Agronegócio – LNNA, Embrapa Instrumentação, São Carlos, SP, Brasil*
*Corresponding author: odilio.assis@embrapa.br

Brazil (latitude: 21°13'33"S, longitude: 43°46'25"W). The apiary was fed with "alecrim-do-campo" (*Baccharis dracunculifolia*), an important plant source of Brazilian green propolis.

Extracts were prepared according to the methodology patented by Embrapa (Brandão, 2013). Briefly, the crude propolis was ground using a mortar into a fine powder and the extracts prepared by mixing lots of 13.75 g into 100 mL of five different water/ethanol proportions: 100/0, 70/30, 50/50, 30/70 and 0/100. Each formulation underwent moderate shaking for 15 days at room temperature and in darkness. After resting and spontaneous precipitation the extracts were filtered in 0.2 μm pore size PVDF membranes (Milipore, MA, USA) and identified as WPE (water pure propolis extract), EPE30 (ethanolic propolis extract at 30% ethanol) and EPE50, EPE70, EPE for ethanol concentration of 50, 70 and 100% respectively.

2.2 Characterization of total phenolic and flavonoid contents

The total content of phenolic compounds in the extracts was determined using the Folin-Ciocalteu method, according to the Zheng & Wang (2001) procedure. A sample (150 μL) was diluted in 2.4 mL of deionized water and mixed with 150 μL of Folin–Ciocalteu reagent at 0.25 mol L^{-1} and allowed to stand for 3 min at 28 ± 2 °C. 300 μL of saturated sodium carbonate solution (Na_2CO_3) was then added to the mixture and allowed to stand for another 25 min at 28 ± 2 °C in the dark. The absorbance was read at 725 nm in a Shimadzu spectrophotometer (Shimadzu model 1600). Gallic acid (Sigma, USA) was taken as a standard for preparing a calibration curve over the concentration range 25-200 mg L^{-1}. The total phenolic content in each extract was determined using the regression equation from the calibration curve as $y = 0.0036x - 0.0214$, $r^2 = 0.99$.

The total flavonoid content was determined according to Funari et al. (2007) method. Briefly, 1 mL of each extract was mixed with a same volume of an ethanolic solution of 2% aluminum trichloride ($AlCl_3$). The mixture was allowed to rest for 30 min in darkness at 28 ± 2 °C and absorbance readings were taken at 425 nm (Shimadzu, spectrophotometer model 1600). A blank sample consisted of 1 mL of $AlCl_3$ with 24 mL of absolute ethanol. The total flavonoid content was determined using a standard curve with quercetin (Sigma, USA) within the concentration range 2 to 12 mg L^{-1} (calibration curve: $y = 0.0781x - 0.0036$, $r^2 = 0.99$). All experiments were performed in triplicate.

2.3 Test bacteria and MIC determination

Two microbial strains were used as test organisms: Gram-positive *Staphylococcus aureus* (ATCC25923) and Gram-negative *Escherichia coli* (ATCC25922). The bacteria were incubated overnight, under aerobic conditions in a Müller-Hinton broth (Merk) at 35.0 ± 0.5 °C. The cells were then adjusted to a 1.5 x 10^8 CFU/mL.

The minimum inhibitory concentration (MIC) was determined by the dilution method in Müller-Hinton broth for both strains. An initial aliquot of 100 μL of each extract was separately mixed with 100 μL of broth followed by a serial two-fold dilution using a 96 well-cell culture microplates (Fisher Scientific, Atlanta). Absolute ethanol was also assayed to check any possible activity of the solvent. The microplates were incubated under aerobic conditions at 35.0 ± 0.5 °C for 24 h. 2, 3, 5-triphenyltetrazolium chloride (TTC) at 1% was added to facilitate bacterial growth visualization. MIC was defined as the lowest concentration of extract that allowed for no visible growth of microorganisms. All measurements were performed in triplicate.

2.4 Preparation and analysis of AFM samples

The effect of the most effective extract (lower MIC) on the bacterial cell surfaces was investigated by atomic force microscopy (AFM) following the Santana et al. (2012) procedure. Both bacteria were inoculated in Müller-Hinton broth overnight at 35.0 ± 0.5 °C in falcon tubes. These were then centrifuged at 2 x g for 15 min at room temperature and washed three times in a saline solution of 0.9%. Then 10^7 UFC mL^{-1} was resuspended in 5 mL of the same saline solution. For propolis treatments, aliquots corresponding to the MIC extract concentrations were added and allowed to interact for 4 and 12 h at 35.0 ± 0.5 °C under agitation. Cell suspension in saline solution (without propolis extract) was used as a control.

After interaction, the suspensions were pelleted by centrifugation (6150 x g) for 15 min, washed and 20 μL samples collected from each treatment and applied onto ultrasonically cleaned (with isopropanol for 10 min) glass slides. The slides were air dried and AFM scanned in intermittent contact mode (frequency near 190 kHz resonance) in a VEECO microscope, Nanosurf model (Santa Barbara, CA, USA). Images were analyzed and processed using Gwyddion Software (version 2.42). The numerical dimensions represent the average of 30 scanned cells.

2.5 Statistical analysis

All data is expressed as mean ± standard deviation. Data was analyzed using one-way ANOVA using Microcal OriginLab v.9.0 software. Significant differences were considered at the level of $p < 0.05$.

3 Results and discussion

3.1 Phenolics and flavonoids content in propolis extracts

The amount of ethanol has significant influence on propolis tincture as can be visually observed by the liquid turbidity (Figure 1). When the ethanol (non-polar solvent) concentration is increased the extract became darker, indicating differences in the amount of solubilized substances.

The total phenolic content in these extracts (as determined spectrometrically) is shown in Figure 2. The highest amount of these compounds was found in EHP50 extract, which solvent has the same polar (water) and non-polar (ethanol) volume proportion. As the ethanol increases in the extract, a reduction in total phenolic concentration was recorded, within the adopted statistical significance ($p < 0.05$). This numerical relation suggests that the raw material contains a slight higher proportion of polar than non-polar phenolic compounds where the highest value,

measured for 50% water/50% ethanol, indicates the partial sum of both solubilized (polar and non-polar) fractions in this extract. Similar concentration proportions were obtained in extracts presented by Miguel et al. (2014).

This result is reasonably consistent with that presented in the literature, in which the twenty most frequent phenolic compounds reported as found in propolis extracts are: caffeic acid, p-coumaric acid, quercetin, pinobanksin, chrysin, galangin, pinobanksin 3-acetate, phenethyl caffeate, cinnamyl caffeate, tectochrysin, 2,2-dimethyl-6-carboxyethenyl-2H-1-benzopyran, 3-prenyl-4-hydroxycinnamic acid, gallic acid, 3,4-dimethoxycinnamic acid, pinobanksin 5-methyl ether, apigenin, kaempferol, cinnamylideneacetic acid, pinocembrin and artepillin C (Marcucci et al., 2001; Kumazawa et al., 2004; Moreira et al., 2008). Of these, the first thirteen are totally or partially soluble in water and the remainder more soluble in alcohol. According to Fischer et al. (2010) artepillin C (phenolic with strong non-polar characteristics) is, however, the most common and present in high relative proportions in most of Brazilian propolis. The estimation of total flavonoid content in the extracts is also presented in Figure 2.

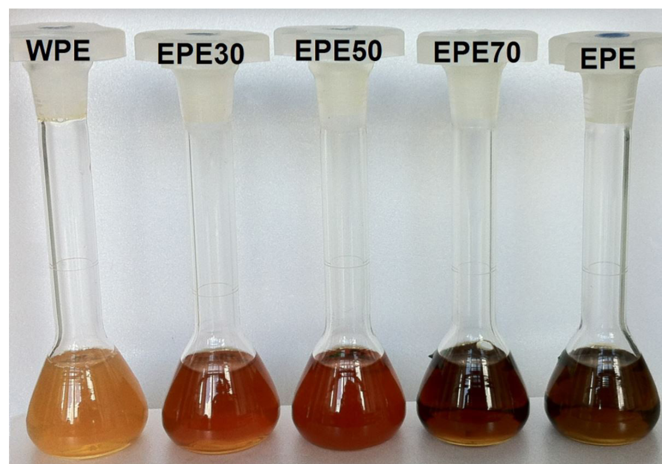

Figure 1. Visual aspects of different extracts of propolis. WPE (water pure propolis extract), EPE30 (ethanolic propolis extract at 30% ethanol) and EPE50, EPE70, EPE for ethanol concentration of 50, 70 and 100% respectively.

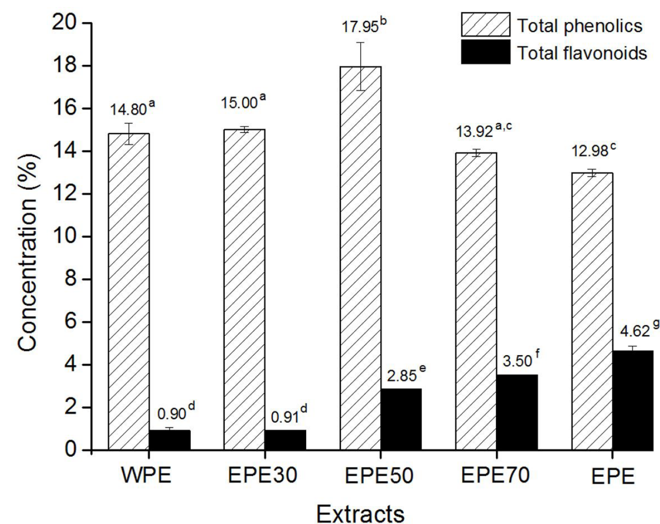

Figure 2. Total amount of phenolic and flavonoid compounds in propolis extracts, expressed in percentage. Different superscript letters in the same class of bar are statistically different at $p < 0.05$.

For these it is evident that the extraction is greatly improved as solvent polarity is reduced (increasing of ethanol concentration). This indicates that the major fraction of flavonoid constituents in the raw green propolis is predominantly non-polar, mainly polyphenols with low molecular weights such as pinobanksin, kaempferol, apigenin, pinocembrin, chrysin and galangin (Hayacibara et al., 2005). Such solvent polarity dependence has been confirmed by analysis, as performed by Mello et al. (2010) who reported that the solubility of flavonoids presented in raw propolis is estimated to be approximately three times greater in ethanol than the fraction soluble in water.

For Brazilian green propolis, the numerical amounts of flavonoids determined in the present study as shown in Figure 2, are in full agreement to the data found in the literature (Funari & Ferro, 2006; Batista et al., 2012).

3.2 Minimum Inhibitory Concentration (MIC) determination

MICs values of propolis extracts against the tested strains (*S. aureus* and *E. coli*) are displayed in Table 1. Initially the effect of pure ethanol on the growth of both bacteria was investigated. For comparison reasons the same mass relation was adopted (mg/mL) for ethanol dilution in deionized water (Lu et al., 2005).

The measured MICs confirm the ethanol as an antimicrobial agent, more effective against Gram-positive than Gram-negative strain, which should be considered in the interpretation of the results. All other extracts showed some *in vitro* activities, with exception of WPE for which no effect (no inhibition) was observed against both bacteria. The effect of ethanol is directly related to the MICs measured for EPE30. WPE and EPE30 have statistically the same amount of phenolic and flavonoid compounds (Figure 2), nevertheless EPE30 has 30% of ethanol in its composition. WPE presents no activity while EPE30 resulted in MIC of 34.68 mg/L for *S. aureus* and 68.75 mg/L for *E. coli* which can be attributed to the action of the ethanol. As summarized in Table 1, absolute

Table 1. Minimal inhibitory concentration (MIC) of propolis extracts against *S. aureus* e *E. coli* strains, in mg/L.

Strain	*WPE*	*EPE30*	*EPE50*	*EPE70*	*EPE*	*Absolute ethanol*
S. aureus	*INO**	*34.38*	*2.15*	*0.54*	*0.13*	*4.30*
E. coli	*INO**	*68.75*	*34.38*	*8.59*	*4.30*	*8.59*

*INO = Inhibition not observed; WPE = water propolis extract; EPE30 = ethanolic propolis extract at 30% ethanol; EPE50, EPE70 and EPE (ethanol concentration of 50, 70 and 100% respectively).

ethanol by itself showed to have superior antibacterial activity than water-based propolis extracts.

In general, the results reveal a direct dependence between bacterium specie and formulation compositions with better activity against *S. aureus* confirming previous studies that have reported propolis to be more effective against Gram-positive bacteria than Gram-negative ones (Silici & Kutluca, 2005; Uzel et al., 2005). Regarding the absence of activity observed in aqueous propolis extract, it can be understood in terms of the low capability of pure water to extract non-polar compounds, since these are stated to be the main responsible for the propolis antimicrobial activity (Nina et al., 2015). The combined effect of propolis and ethanol has the lowest MIC values for both tested bacteria, as recorded for EPE. This is understood as resulted from a synergistic interaction of ethanol and the extracted non-polar compounds, mainly flavonoids, as concentrations presented in Figure 2.

In Figure 3 is shown representative atomic force microscopy images of the control and microorganisms exposed for 4 and 12 hours to EPE. *E. coli* control cells have a characteristic rod shape with average dimensions around 2.0 × 0.8 × 0.3 µm (length × width × height) arranged singly or in pairs. The cells before treatment have a homogeneous surface in all directions without any visible damage. In some samples it is possible to identify the presence of fimbria and pili structures although it is known that the centrifugation process causes the break of the filaments from the cells (Tripathi et al., 2012). Microorganisms exposed to 4 h (b) suffer an overall increase in dimensions, presumably due to changes in the permeability of the outer membrane favoring internal water uptake via an osmotic process. In treated samples the swelling is evident with loss of shape and surface smoothness. More significantly is the effect after 12 h interaction (Figure 3c) in which it is clearly observed the presence of disruptions in the outer membrane, confirming that binding to lipid bilayer sites is one of the targets of the propolis

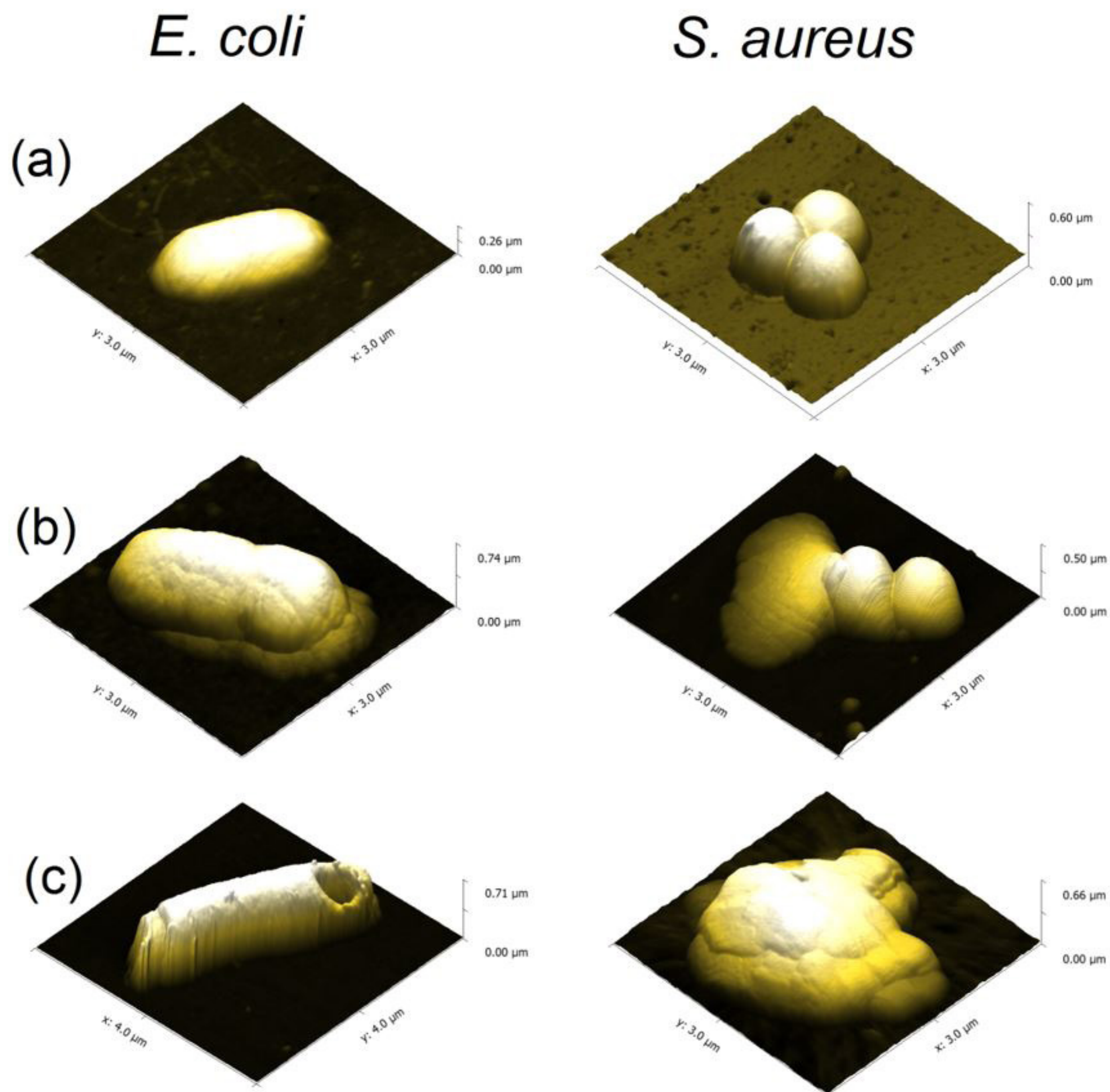

Figure 3. Typical 3D AFM images of *E. coli* and *S. aureus*, before (a) and after exposure to EPE for 4 (b) and 12 hours (c).

compounds. These observations suggest that the antimicrobial action could be ruled by electrostatic-based intermolecular interactions. In the surface of Gram-negative bacteria anionic molecules are oriented towards the exterior (Silva & Teschke, 2003) while the propolis is characterized as having predominant cationic groups (Figueiredo et al., 2014).

For *S. aureus*, images of control microorganisms reveal typical cocci (near spherical-shaped cells), mostly arranged in clusters. After 2 hours of interaction, some cells have morphological alterations, changing from round to a more spread-like shape with volume increasing. AFM profile analysis (Figure 4) indicates possible occurrence of internal contents leaking out accompanied by cell swelling. This is probably due to water uptake through cell lysis. For cells analyzed after 12 h treatment (Figure 3c), all organisms in the clusters showed completely collapse with deep changes in their format. Nevertheless, no rupture is clearly evidenced for *S. aureus* as that observed for *E. coli* after 12 h treatment.

The morphological alterations are accompanied by differences in numerical measurements. Results of dimensions and volume are reported in Table 2. From these it is possible to quantify the differential entropy of the value distribution using the Gwyddion Software. The differential entropy is a useful parameter to compare loss of similarities (measure of randomness) amongst samples as a result of different treatments (Bitler & Dover, 2012). The analyses point that both bacteria resulted in no significant statistical differences of geometric entropy values over time, indicating that the effect of extracts exposure occurs similarly either in Gram-positive or in Gram-negative tested strain.

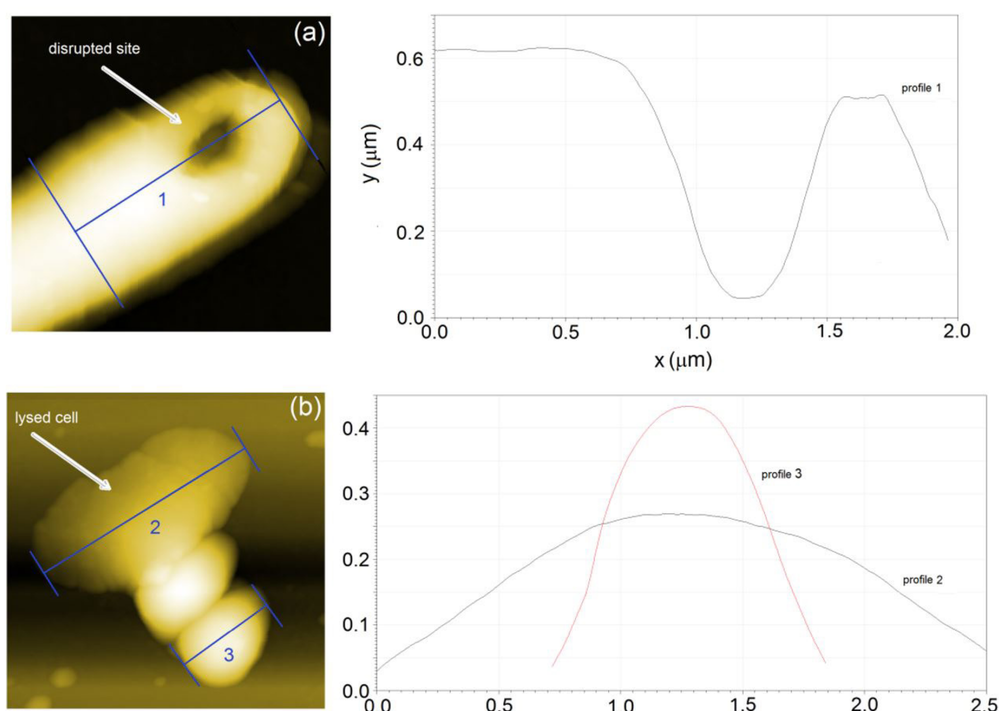

Figure 4. AFM two-dimension images of *E. coli* (a) and *S. aureus* (b) cells and respective topographic profiles. The arrows indicate ruptured and lysed cells.

Table 2. Morphological measurements of control and treated cells as average of 30 scanned samples.

	Escherichia coli		
	Control	4h treatment	12h treatment
volume (µm³)	0.30 ± 0.04ᵃ	1.66 ± 0.47ᵇ	1.400 ± 0.44ᵇ
height (µm)	0.337 ± 0.07ᵃ	0.69 ± 0.06ᵇ	0.74 ± 009ᵇ
length (µm)	2.05 ± 0.03ᵃ	2.83 ± 0.58ᵇ	2.87 ± 0.78ᵃ
diameter (µm)	0.81 ± 0.18ᵃ	1.40 ± 0.15ᵇ	1.02 ± 0.22ᵃ
entropy	-18.01 ± 0.32 ᵃ	-17.62 ± 0.22 ᵃ	-16.08 ± 0.61 ᵃ
Staphylococcus aureus			
volume (µm³)	0.25 ± 0.11ᵃ	0.34 ± 0.11ᵇ	0.75 ± 0.19ᵇ
height (µm)	0.53 ± 0.08ᵃ	0.63 ± 0.09ᵇ	0.67 ± 0.06ᵇ
diameter (µm)	0.96 ± 0.12ᵃ	1.22 ± 0.20ᵇ	1.54 ± 0.40ᵇ
entropy	-18.08 ± 0.44 ᵃ	-17.05 ± 0.07 ᵃ	-16.89 ± 0.42 ᵃ

Means in the same line followed by different letters indicate significant statistical difference at $p < 0.05$.

4 Conclusions

From the results of this study it can be concluded that the concentration of flavonoids and phenolic compounds in propolis extracts determine antibacterial activity. The most likely antimicrobial mechanism is based on electrostatic interaction between the main cationic moieties (positively charged groups) of the extract compounds and the bacterial surface anionic sites (negatively charged). Such interaction is clearly time-dependent and leds to damage of the cell walls. Both Gram-positive and Gram-negative bacterium suffers some kind of cellular damage with loss of the original form. According to AFM image analysis there is an increase of dimensions of treated microorganisms, interpreted as an osmotic water uptake due to lyses in the cellular walls. Comparatively, the Gram-positive *S. aureus* bacteria exhibited less pronounced damages, attributed to differences in the structure of cell membranes.

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